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Paul S. Cremer

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Texas A&M University
Department of Chemistry 3255 TAMU College Station, TX 77843

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14. ABSTRACT

Two single molecule fluorescent microscopes were designed and built for exploring membrane protein assembly. The first system, which was designed for use with solid supported membranes, is being used to explore the mechanistic details of alpha-hemolysin assembly. On the other hand, the second system was fabricated for use with blacklipid membranes. It is being employed to correlate ion channel insertion into bilayers with changes in current. he first results with these systems already show great promise.

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single molecule detection, fluorescence microscopy, membrane protein assembly.

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Final Report

Grant #: N00014-02-0710

Principle Investigator: Paul S. Cremer

Institution: Texas A&M University

Grant Title: Single-Molecule Detection of Proteins in Supported Bilayers: Applications in Device Fabrication and Sensor Technology

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Objective: To design and build two state-of-the-art single molecule fluorescence microscope setups for monitoring membrane protein assembly in a phospholipid bilayer. One apparatus was specifically designed to work in combination with solid supported membranes, while the other was designed for use with black lipid membranes.

Approach: Inverted fluorescent microscopes were designed to work in total internal reflection (TIR) or epifluorescence mode using mixed gas (krypton and argon) ion lasers. TIR was performed inside the objective itself. The systems were set up on standard laser tables. Both fiber optics and standard flat mirror optics were employed to deliver the collimated radiation to the sample. The latter worked best when different excitation wavelengths were desired, while the former proved to be more convenient for a single wavelength of interest. The I-PentaMAX with a preamplifier was used to collect fluorescent images. This could be done with frame rates approaching 1 kHz, although there was a direct tradeoff between frame rate, pixel resolution, and sensitivity.

Accomplishments: The first system, which was designed for use with planar supported phospholipid bilayers, was completed in the spring of 2003 and the preliminary results now being obtained are quite promising. In a first set of experiments, the system was used to measure the diffusion constant of α-hemolysin (both in monomer and heptamer form) in a phospholipid bilayers at the single molecule level. For this purpose Alexa594-labeled K288c mutants were employed. The protein monomers (10 ng/ml) were incubated above a glass substrate coated with an Egg PC bilayer for 30 minutes. At this

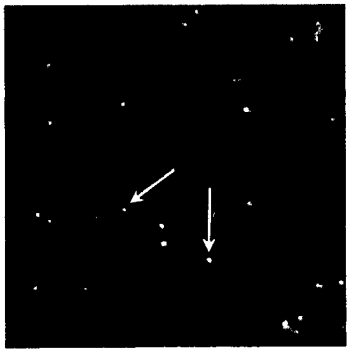


Figure 1. Fluorescence image of Alexa594-labeled K288c mutants of α-hemolysin on Egg PC bilayers. The bright spots in the image (two white arrows shown as examples) are the protein monomers. The image is 12 x 12 μm.

point excess protein was washed away and the sample was placed under the microscopy setup and imaged (Figure 1). The monomers were indeed mobile at the interface as determined by time-lapsed imaging. On the other hand, control experiments performed without coating the surface with a lipid bilayer gave rise to only immobile species. The

protein monomer movement could be tracked as a function of time and the results are plotted in Figure 2a. Data from a few dozen monomers were tracked in a similar fashion. This allowed the mean-square

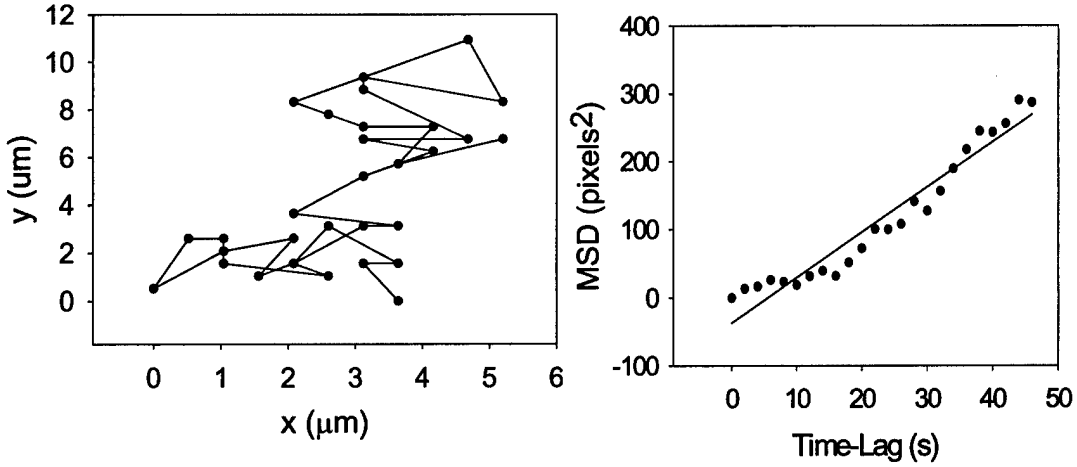
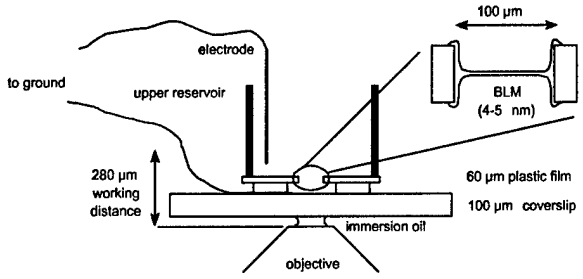


Figure 2. (a) Random walk of a hemolysin monomer on a Egg PC membrane. (b) Plot of the average mean squared displacement (MSD) of a few dozen hemolysin molecules.

displacement of these molecules to be calculated as a function of time (Figure 2b). The results plot to roughly a straight line with a slope of 0.2 μm^2/sec. This number is taken as the diffusion constant for the single molecule measurements. With the ability to make single molecule tracking measurements in hand, it should now be possible to extend the technique to mechanistic studies of membrane protein assembly.



The second system is designed to be used with planar lipid bilayers.

Fig. 3 Single-Molecule Fluorescence of Planar Bilayers.

This purpose required development of an approach to construct black lipid membranes (BLMs) within the short working distance (~280 μm) of a high power (100X) objective. We have found that these bilayers can be routinely and quite easily made by the brush technique using the basic set-up shown in Fig. 3. The bilayer itself can be visualized with transmitted light as a thin circular region in the center of the ~100 μm aperture with an annulus that contains residual lipid and organic solvent (Fig. 4). Simultaneous

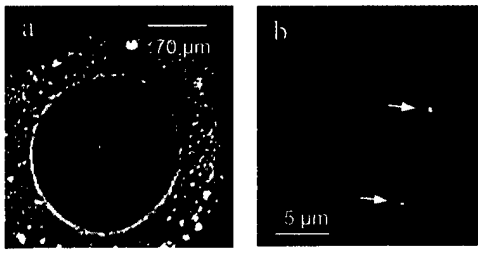


Fig. 4 SMF in Planar Lipid Bilayers (a) Transmitted light image of planar bilayer. (b) SMF of Alexa555-hemolysin on planar bilayer.

single channel recording confirms the presence of the bilayer and the insertion of single membrane channels. We currently still have difficulties with noise from particulate matter, but this should be resolvable.

We have multiple experimental systems that we plan to examine with this system:

• **The assembly pathway of α-hemolysin**: α-Hemolysin (αHL) is a staphylococcal exotoxin. The monomeric 293-amino-acid polypeptide assembles into a heptameric pore, in which a transmembrane β barrel is formed by the central residues of the polypeptide chain. The assembly pathway of the αHL pore was sketched by biochemical, biophysical and molecular genetic experiments, largely by the Bayley and Gouaux groups (Figure 5).

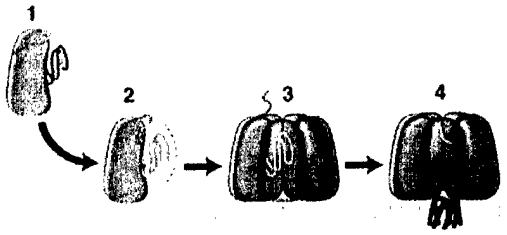


Figure 5. Membrane-bound αHL monomers (2) associate to form a heptameric "prepore" (3), oriented with its 7-fold axis perpendicular to the membrane surface. In forming the fully-assembled SDS-resistant heptamer (4), the glycine-rich central domains of the seven subunits penetrate the lipid bilayer to form the transmembrane barrel.

In the research underway here, we are elucidating further details of the assembly process by using single molecule fluorescence to track binding, diffusion, oligomerization and insertion of the αHL polypeptide.

Examples of questions that we are tackling by tracking and characterizing individual fluorescent particles include: Is the addition of subunits into the growing oligomer sequential (1→2→3→... 7) or random (e.g. 1+2→3, 2+3→5 ... 7)? Is the insertion of subunits into the membrane concerted or sequential? The fine details of a pathway with multiple substeps in which fast changes may follow slow ones are only accessible at the single molecule level. The experiments are being performed on supported bilayers. In the initial experiments, binding and diffusion are being monitored by FRAP (fluorescence recovery after photobleaching: an ensemble experiment), fluorescence correlation and by tracking individual dye molecules. We are using mutant proteins that can be arrested at each stage in assembly, as well as pre-assembled intermediates, to help interpret the observations made after the addition of fluorescent wild-type monomers to bilayers. At this point, we have constructed the following dye-labeled proteins.

T129C-Alexa555: expected to act like the wild-type protein.

H25W-T129C-Alexa555: expected to bind to membranes, but become arrested there as a monomer (2).

H5- T129C-Alexa555: expected to become arrested as the prepore (3) in the presence of Zn^{2+} ions. Can be released for the final step of assembly (3 → 4) with EDTA.

(T129C-Alexa555)$_7$: preformed heptamers (4). These molecules will insert into bilayers to yield a population of heptamers.

After the initial experiments are complete, we will monitor oligomerization through the time dependence of fluorescence intensity histograms, observing step-changes in fluorescence of individual particles and by watching protein-protein interactions by single-molecule fluorescence energy transfer (FRET). Finally, conformational changes that occur during assembly, notably that of the prepore to pore transition, will also be detected by FRET, by using monomers doubly-labeled with dyes with the appropriate R_O

values. The membrane insertion of the central domain of individual subunits will also be observed at the single-molecule level through blue shifts in the emission of individual dye molecules. Assembly takes place on a time-scale of minutes. Therefore, it will easily be possible to resolve the events described above.

• **E. coli Sec and Tat machineries** – The *E. coli* Sec and Tat machineries transport proteins across the periplasmic membrane without collapsing the transmembrane electric and proton gradients (pmf = proton motive force). Our goal is two-fold.

First, we wish to use the planar bilayers as a model system to examine the Sec and Tat transport process using single molecule fluorescence (SMF). In the case of the Sec machinery, two soluble factors (SecA and SecB) are required for transport. We will determine when in the transport cycle these proteins are released from the translocon/substrate protein complex. For the Tat machinery, we plan to monitor assembly of the translocon from individual TatA and TatB/C molecules. For both translocons, we will first determine the total transport time, and then break this down into individual kinetic constants by appropriate use of FRET pairs and mutant proteins. We have constructed a fluorescent Sec substrate and demonstrated that it is transport competent. Purification of a fluorescent Tat substrate is almost complete. Also, we are in the process of determining the best means of fusing native or reconstituted membrane vesicles with these bilayers.

Second, while the pmf is maintained during protein transport through both of these machineries, substrate transport induces additional ionic leakage through the membrane. Simultaneous SMF and single channel recording measurements will allow us to measure the extent of this leakage. This is expected to be a many channel (>100) rather than a single channel experiment since the leakage per channel is much less than the current from single ion channels. SMF will allow us to estimate the number of active channels. In addition, the electrophysiology equipment will provide and easy means to set and maintain the membrane potential and determine its effect on transport rate and efficiency.

Conclusions: Simultaneous SMF and single channel recording measurements are possible and can provide a wealth of heretofore inaccessible information on the properties of membrane proteins

Significance: This powerful technology opens up a new area of investigation for membrane proteins in general, and channel proteins in particular.

Patent Information: NA

Award Information: Camille Dreyfus Teacher-Scholar Award (2003), Alfred P. Sloan Research Fellowship (2002), and my co-PI also won a major grant, NIH R01 GM065534 (Musser).

Publications: It should be noted that since this was a DURIP award (duration 1 year), publications with these instruments are not yet available. It took over six months to order, receive, assemble, and test these new devices. We do, however, expect many publications with these instruments over the next decade.